Vagal stimulation induces expiratory lengthening in the in vitro neonate rat

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Mellen, Nicholas M., and Jack L. Feldman. Vagal stimulation induces expiratory lengthening in the in vitro neonate rat. J. Appl. Physiol. 83(5): 1607–1611, 1997.—Respiration is modulated by lung mechanoreceptor feedback in vivo on a cycle-to-cycle basis. We replicated this modulation in vitro and tested four stimulus protocols to identify which of these most closely replicated in vivo responses to lung mechanoreceptor activation in mammals. We activated pulmonary vagal afferent pathways by electrical stimulation or by lung inflation, applied during expiration, which produces expiratory lengthening in vivo. In each modality, transient and tonic stimuli were applied. Stimuli were applied over a range of delays following inspiratory termination. Tonic stimuli were maintained until subsequent inspiratory onset. All stimulus modalities prolonged expiration (P < 0.05). These results indicate that the neural circuitry mediating pulmonary afferent modulation of expiratory duration is retained in vitro.

In intact mammals, ventilation results from a centrally generated rhythm processed through premotor circuits and modulated by afferent feedback. Although the effects of pulmonary afferent feedback are well characterized in vivo, their cellular basis is difficult to study because of interaction between various afferent inputs and the difficulty in accessing and manipulating relevant neuronal populations. By extending experimental protocols from in vivo to in vitro, novel investigation of the cellular and synaptic basis for respiratory rhythm generation is made possible (7, 12). We sought to extend the in vitro approach to understanding pulmonary afferent modulation of respiratory pattern by reproducing in vivo pulmonary reflexes in vitro. To do this, we used a lung-attached neonate rat brain stem/spinal cord preparation developed by Murakoshi and Otsuka (10).

The effect of lung inflation on respiratory pattern varies with respiratory phase in vivo (Hering-Breuer reflex; see Refs. 5, 6). Lung inflation during inspiration terminates inspiratory activity, early in expiration lengthens expiration, and late in expiration has no effect on expiratory duration. Similar effects are produced by moderate electrical stimulation of the vagus nerve (1, 4, 6).

The Hering-Breuer reflex is mediated by slowly adapting pulmonary mechanoreceptors (SARs). SARs are inflation sensitive, respond briskly to changes in lung pressure, and fire tonically in relation to lung volume (1). SARs are part of the vagus (X) nerve projecting to the medial nucleus tractus solitarii (3).

In an in vitro neonate rat brain stem/spinal cord with lungs attached, inspiratory activity is delayed when lungs are inflated during expiration (10). In these experiments, pressure changes outside of the physiological range (20 cmH2O) were used to inflate the lungs. We used a similar procedure, but with pressure changes in the physiological range (3–8 cmH2O) (9). We compared changes in expiratory duration resulting from lung inflation with changes in expiratory duration resulting from electrical stimulation of the vagus nerve. We also compared stimuli with a tonic component to transient stimuli. In this study, we did not investigate responses to lung inflation during inspiration.

We found that sustained lung inflation within the physiological range and electrical stimulation reliably gave rise to expiratory lengthening. Transient lung inflation was least effective in changing expiratory duration. Our results suggest that the neural substrate mediating the Hering-Breuer reflex in vivo is retained in the in vitro preparation. Some of the results have been published in abstract form (8).

METHODS

Dissection. Six Sprague-Dawley rats (newborn to 4 days old) were used. The basic dissection methods are described elsewhere (11). The preparation was modified so as to retain lungs, heart, and trachea, connected to the brain stem by the right vagus nerve (Fig. 1A). The left vagus nerve was transected close to the point of entry into the lung and used for electrical stimulation.

The C1 ventral root was drawn into a glass suction electrode for recording, and the left vagus nerve was drawn into another suction electrode for stimulation. A cannula (22-gauge) was inserted into the trachea, held in place with a suture, and connected to a computer-controlled precision syringe pump (Carnegie Medecin M100) for controlled lung inflation. Lungs and cannula were filled with saline (27°C). Pressure changes were monitored by measuring changes in the height of a column of saline connected to a branch off the syringe pump.

Experimental protocol. To investigate the phase dependence of the response to SAR stimulation, stimuli were applied at various delays relative to inspiratory onset (Fig. 1B). Response to a stimulus was measured as a change in pressure, defined as the interval between consecutive inspiratory bursts (Fig. 2A).

We used two stimulus modalities: lung inflation or electrical stimulation of the cut vagus. Stimulus protocol parameters were 1) delay following inspiratory onset, 2) number of control cycles between stimuli, and 3) stimulus magnitude (pressure or current).

Constant-current electrical stimuli (1–15 µA) ranged in amplitude between 4 and 8 mV. We measured the volume of fluid injected into the lungs as well as the resultant pressure change. Volumes injected ranged between 0.4 and 0.8 ml, which corresponded to a pressure change of 3.0–8.0 cmH2O.
and produced uniform inflation of lungs. In both modalities, stimulus amplitude was the minimum at which lengthening was obtained in comparison to control periods, when applied at midexpiration.

Transient stimuli were compared with stimuli with a tonic component. In the lung inflation protocol, transient stimuli were obtained by lung inflation immediately followed by deflation to resting volume (Inflate/Deflate); stimuli with a tonic component were obtained by inflating the lung and holding it inflated until the subsequent inspiratory burst (Inflate/Hold). In the electrical stimulation protocol, transient stimuli were obtained by applying a brief, high-frequency electrical stimulus (Burst; 8–12 pulses, 5-ms duration, 100 Hz); stimuli with a tonic component were obtained by a brief, high-frequency train followed by a sustained low-frequency train, terminating at the subsequent inspiratory burst (Burst/Tonic; high-frequency train: 8–12 pulses, 5-ms duration, 100 Hz; low-frequency train: 5-ms pulses at 33 Hz). Stimulus protocols are summarized in Fig. 1B.

We use the term "bout" to denote the repeated application of a given stimulus type over a range of delays. Each experiment consisted of multiple bouts. In each bout, stimuli were applied at fixed delays after inspiratory onset. At least five control cycles separated cycles with stimulus application.

Between bouts the preparation was left undisturbed for at least 20 cycles. Up to seven bouts occurred per experiment. The order of stimulus types in consecutive bouts was random.

Stimulus delays spanned as much of the expiratory phase as possible, based on an estimate of respiratory period from control cycles collected before onset of stimulus application. No stimuli were applied later than at 88% of mean control expiratory duration. The briefest stimulus onset delays were 1 s after inspiratory offset. For each bout, stimuli were applied for up to four different delays, evenly spaced between shortest and longest, and 2–15 repetitions of each stimulus delay were applied.

Data acquisition, real-time signal processing, and stimulus control. Respiratory-related motoneuronal activity recorded from C1 was amplified and filtered by using a low-noise amplifier (band pass = 100–10 kHz, gain = 50,000 times) and stored on tape. After being full-wave rectified and integrated (time constant ≈ 20 ms), activity was digitized by using a workstation (VAXLab 3200). The computer detected and stored inspiratory burst onset times and displayed cycle period in real time. In addition, the computer controlled the syringe pump or stimulator, applying stimuli at appropriate delays. All software was developed by using the RTI graphical programming interface (Kinetic Systems).
Data analysis. Periods and stimulus delays were normalized by using the mean of control cycle periods from within each bout. Normalized data were pooled across bouts and experiments. Stimulus effect as a function of stimulus delay was tabulated by sorting normalized stimulus delays into four bins: 0–0.2, 0.2–0.4, 0.4–0.6, and 0.6–1.0 (Fig. 1B). Test cycles associated with stimuli were sorted into bins as a function of the stimulus delay.

For each bout, we tested for stimulus effects on cycles subsequent to the perturbed cycle by using paired t-tests between normalized unperturbed periods, indexed sequentially from the perturbed period. Because we calculated multiple t-tests, we applied Bonferroni correction on the computed P values. For slow time-course changes in respiratory rhythm within a bout, we compared the initial control cycles of each bout with control cycles of subsequent bouts by using single-factor analysis of variance (ANOVA) implemented with Excel (Microsoft).

We assessed whether stimulus effect varied as a function of stimulus modality or delay by using two-way ANOVA. Because the datasets were of unequal size, we used the general linear model module in SAS. The normalized perturbed periods for a given bout were divided into five bins as a function of delay, and the average for each bin in a given bout was the unit of analysis, with normalized control periods for each bout (average = 1.0) included as well. In the ANOVA analysis, two factors were considered: stimulus type and delay bin. Factor delay bin had five categories, the elements of which were the means of perturbed periods for that bin from all bouts (categories 1–4) as well as the means of control cycles (category 0). Factor stimulus type categorized bin means for each bout according to stimulus type (categories 1–4) and included control cycles (category 0). We compared means post hoc using the Tukey least significant difference criterion for significance for all combinations of stimulus type and delay bin.

RESULTS

All stimulus modalities prolonged expiration. Control cycles were normally distributed, whereas perturbed periods appeared bimodal, with the lower mode overlapping with the control period distribution. There is little overlap between the histograms of stimulus delays and control period (Fig. 2B), confirming that stimuli were applied within expiration.

We tested whether stimulus effects persisted into the cycles after their application by comparing each period immediately following a stimulus application to all other unperturbed periods following that stimulus application (Fig. 3A). In 3 of 26 bouts (Burst/Tonic: 2 occurrences, and Inflate/Deflate: 1 occurrence), the period following the perturbed period was significantly different from the other unperturbed periods (P < 0.05).

We tested for slow time-course changes in respiratory frequency over the course of an experiment by carrying out t-tests, in which the first 20 control periods of a bout were compared with 20 control periods from the preceding bout, and we found no significant differences (P ≥ 0.12). Control periods collected over 90 min are shown in Fig. 3B. A histogram of mean control periods is shown in Fig. 3C.

To assess the effect of stimulus delay and stimulus modality on perturbed period, we plotted normalized perturbed periods as a function of stimulus delay (Fig. 4). Across stimulus modalities, the effects of stimuli were variable, with a subset of test cycles in most delay bins for all stimulus modalities clustered at 1.

We tested whether control cycles were significantly different from perturbed cycles and whether perturbed cycles were significantly different as a function of stimulus delay or stimulus modality by using repeated-measures ANOVA with two factors: stimulus type and stimulus delay (Fig. 5). For both factors, the difference between control and perturbed periods was statistically significant (P < 0.01). Within stimulus type, all stimulus modalities were significantly different from control. Of all stimulus types, Inflate/Deflate elicited the weakest response, and means from bin 3 were not significantly different from control. For factor stimulus delay, bins 1–4 were found to be significantly different from each other but all were significantly different from control (bin 0). No interaction effects

Fig. 3. A: normalized periods of perturbed and unperturbed cycles from 1 bout (burst stimulus, delay range 0.2–0.7). Perturbed cycles are indicated by arrow; 4 following unperturbed cycles are indexed relative to perturbed cycle. Mean control period for this bout: 7.2 s. B: time-course of control cycle periods from a single experiment, pooled across bouts. C: histogram of control cycle period means for all bouts.
between stimulus delay and stimulus modality were statistically significant.

**DISCUSSION**

Pulmonary stretch receptor modulation of respiratory pattern plays an important role in the regulation of ventilation in both adult and neonatal mammals. We used electrical and mechanical stimuli to activate pulmonary afferent pathways and were able to obtain consistent expiratory lengthening in response to stimuli applied during expiration.

Our results support the hypothesis that the neural substrate associated with the Hering-Breuer reflex in vivo is preserved in a highly reduced in vitro brain stem-lung preparation from neonatal rat. We replicated the finding of Murakoshi and Otsuka (10) that lung inflation during expiration produces expiratory lengthening in the in vitro brain stem/lung preparation from neonatal rat but we did so by using applied pressures in the physiological range (3–8 cmH₂O vs. 20 cmH₂O). In addition, we obtained expiratory lengthening by using electrical stimulation. Thus we can consistently modulate respiratory rhythm by activation of a well-characterized afferent pathway in vitro. The presence of test cycles periods close to 1 (Fig. 4) is consistent either with stimulus amplitudes close to the minimum required to elicit expiratory lengthening or with relatively weak modulation of respiratory rhythm by this afferent pathway in the in vitro preparation.

We did not apply stimuli sufficiently late in expiration to test whether the loss of effect to stimuli applied after 90% of expiration observed in vivo (6, 13) could be reproduced in vitro. We avoided stimuli applied close to the expected onset time of the next inspiratory burst for two reasons: 1) to avoid false positive responses for electrical stimuli, which could arise because inspiratory burst detection was disabled during stimulus application to mask stimulus transients; and 2) to avoid false negative responses for mechanical stimuli, which would occur if over the course of stimulus application (0.3–0.8 s) inspiratory onset preceded mechanoreceptor activation.

Although both mechanical and electrical stimuli gave rise to expiratory lengthening, it does not follow that the observed lengthening was achieved by the same means. As Breuer originally noted (5), electrical stimulation of the vagus nerve activates afferent pathways in a synchronous and nonspecific manner, so that cardiovascular afferents, as well as rapidly adapting mechanoreceptors and C fibers, may all contribute to the response obtained under electrical stimulation. In neonates, this problem may be particularly pronounced, since myelination of vagal fibers may be incomplete, and thus the threshold differences, which allow for the selective activation of myelinated fibers in adults, would not exist.

**Fig. 4.** Binned normalized perturbed periods from all bouts as a function of stimulus delay for each stimulus type. Gray squares indicate mean of perturbed periods for bins 1-4.

Inflated/Deflated produced the weakest expiratory lengthening of all modalities used. By contrast, Inflated/Hold produced robust lengthening of expiration. The difference in response to transient and tonic mechanoreceptor activation suggests that expiratory lengthening obtained by Inflated/Hold is sustained by the tonic firing properties of SAR afferents. Although our preparation differs from in vivo in that afferent feedback is only present in test cycles, we have nonetheless confirmed that the pathway mediating mechanoreceptor afferent modulation of respiratory rhythm is preserved in vitro and responds to pressure changes within the physiological range. This finding
strengthens the link between fictive respiration in vitro and eupnea in vivo.

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